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Article

Bone Morphogenetic Protein 4 Signaling Regulates Epithelial Renewal in the Urinary Tract in Response to Uropathogenic Infection

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SUMMARY

The transitional epithelium of the bladder normally turns over slowly but upon injury undergoes rapid regeneration fueled by basal uroepithelial stem and/or early progenitor cells (USCs). Little is known about the mechanisms underlying the injury response. We investigate the mechanism of bladder epithelial regeneration in response to infection with uropathogenic *E. coli* (UPEC). Infection resulted in rapid sloughing of superficial cells, a marked inflammatory response, and a substantial spike in basal cell proliferation. In mice with induced urothelial ablation of a member of the TGF-β receptor superfamily, bone morphogenetic protein (Bmp)-4 receptor, infection led to aberrant urothelial renewal resulting from a block in USC differentiation into superficial cells. Chemical injury also caused sloughing but no inflammation or USC activation. Together, our study indicates that UPEC infection but not chemical injury activates the USC niche, and Bmp signaling is required for regulation of the USC response to infection.

INTRODUCTION

Self-renewing tissues like skin and intestine contain stem cells (SCs) that provide the tissue's regenerative potential. Adult SCs typically reside in specialized niches where they receive microenvironmental cues that govern normal homeostasis and wound repair (Kobielak et al., 2007). The mammalian urinary bladder is lined by a self-renewing, 3- to 4-cell-layer deep pseudostratified transitional epithelium (urothelium). The urothelium has a slow rate of turnover under physiological conditions (Hicks, 1975), yet maintains a remarkable capacity for regeneration. Adult urothelial cells, when challenged, are capable of completing the cell cycle as fast as SCs in the embryonic bladder (Jost, 1986, 1989), suggesting the presence of an SC population. However, the slow rate of epithelial turnover has hindered the investigation of putative urothelial SCs. In addition, urothelial SCs have not been definitively identified at the ultrastructural level, nor has their proliferative capacity been defined in isolated cell populations, quantified by flow cytometry, or correlated with patterns of marker expression (Huh et al., 2006). Nevertheless, ^{3}H-thymidine labeling (Jost, 1986; Jost and Potten, 1986) and more recently 5-bromo-2′-deoxyuridine (BrdU) label retention assays (Kurzrock et al., 2008) have shown that the proliferative compartment localizes to the basal layer of the urothelium. These basal cells are characterized by small size (5–10 μM), low granularity, and high β4 integrin expression. They demonstrate superior clonogenic and proliferative ability compared to unlabeled epithelial cells (Kurzrock et al., 2008) and specifically express epithelial cell keratins 5 and 6 (Mysorekar et al., 2002), which are basal cell-specific markers in the prostate, lung, and other epithelia (Reis-Filho et al., 2003; Riedel et al., 2001). These cells proliferate and terminally differentiate into large (80–120 μM) and binucleate superficial facet cells that line the luminal surface of the bladder (Hicks, 1975). Herein, we define these basally located cells that take up nucleotide analog upon activation and colocalize with cytokeratins 5 and 6 (Cyt5&6) as the putative urothelial stem and/or early progenitor cells (USCs).

We and others have shown that unlike the undamaged urothelium, which may require up to 40 weeks to renew itself in the adult mouse (Jost, 1989), acute injury caused by infectious or noninfectious agents leads to rapid renewal of the epithelium that can begin within hours of the molecular insult (Mulvey et al., 1998; Mysorekar et al., 2002). However, little is known about the regulatory mechanisms governing USCs in response to infectious diseases that plague the bladder, namely urinary tract infections (UTIs).

UTIs are among the most common infectious diseases in humans, resulting in an estimated 8 million outpatient visits yearly in the U.S., with an estimated cost of evaluating and treating this disease exceeding $1 billion (Foxman, 2003). Uropathogenic *Escherichia coli* (UPEC) are the major causative agents of UTIs. In a murine model of UTI, UPEC infection of the bladder elicits a host response that initiates superficial cell apoptosis concomitant with inflammation. Regeneration of the lost superficial cells proceeds rapidly over a 72 hr period postinfection, with restoration of the intact urothelium by 7 days (Mulvey et al., 1998).

The rapid onset but limited duration of the epithelial renewal process in response to UPEC UTI suggests that regulation of SC proliferation and differentiation in the bladder is tightly controlled. In vitro studies have implicated a potential role for several pathways in urothelial renewal, e.g., TGF-α/-β, EGF and FGF, and KGF families (de Boer et al., 1994, 1996; Daher et al., 2003), but there are few in vivo studies that have attempted to elucidate the signaling governing urothelial regeneration

following injury. A study investigating the host response to UPEC infection using microarrays with cDNA from infected C57BL/6 mouse bladders found prominent changes in the expression of molecular regulators and effectors of epithelial proliferation and differentiation. One key regulator of urothelial proliferation and differentiation was determined to be a member of the TGF-β superfamily of secreted signaling molecules, bone morphogenetic protein 4 (Bmp4) (Mysorekar et al., 2002).

Bmp4 is a key developmentally regulated signaling molecule known to be important for cell survival, proliferation, and differentiation in various embryonic tissues. *Bmp4* is expressed in the developing urinary tract and designates the sites of ureter formation (Miyazaki et al., 2000, 2003). *Bmp4* binds to and signals through two serine/threonine kinase receptors, *Bmpr1a* and *Bmpr1b*. The canonical Bmp signal is mediated by Smad transcriptional factors, which upon activation translocate to the nucleus, where they transactivate their target genes (Mishina, 2003). In the adult mouse bladder, Bmp4 mRNA is localized to the mesenchyme underlying the normal bladder urothelium, and UPEC infection promotes a decline in mRNA levels within this cellular compartment. *Bmpr1a* is the only known receptor for *Bmp4* detectable by quantitative real-time PCR in the bladders of uninfected mice, but expression levels did not change upon infection (Mysorekar et al., 2002). Levels of Phospho-Smad1, the downstream mediator of *Bmp4* pathway activity, were decreased in basal and suprabasal cells following infection, providing evidence for regulation of Bmp4 signaling during a UTI. These results suggested a hitherto unappreciated role for the bladder mesenchymal compartment in inducing or maintaining epithelial renewal.

Urothelial cells express pathogen pattern-recognition receptors such as TLR4 to detect the presence of bacterial lipopolysaccharide (LPS) (Schilling et al., 2001). The secretion of cytokines and chemokines enhances recruitment of inflammatory cells such as neutrophils to the site of infection to remove extracellular bacteria (Mulvey et al., 2000 and references therein). There is little known about how inflammatory processes affect tissue SC niches. However, recent studies have shown that, in colonic epithelia, macrophages can coordinate inputs from luminal microbes and injured epithelium to transmit regenerative signals to neighboring progenitor cells of the colon (Pull et al., 2005).

Here, we show that inoculation of uropathogenic *E. coli* into adult female mouse bladders activates the presumptive USC niche. Next, we show that blocking the transduction of the mesenchymal Bmp4 signal by genetic ablation of *Bmpr1a* in the urothelium results in (1) reduced proliferation of the USC in response to infection and (2) aberrant proliferation of normally terminally differentiated superficial cells. Finally, we show that this inversion of normal patterns of renewal may be dependent on the concomitant induction of an inflammatory response, as noninfectious injury does not elicit activation of the progenitor niche.

RESULTS

E. coli Infection of the Bladder Results in Increased Epithelial Proliferation and Elicits Rapid Epithelial Turnover Fueled by Basal Stem/Progenitor Cells

UPEC invade into terminally differentiated superficial facet cells and rapidly replicate, forming intracellular bacterial communities (IBCs) that have biofilm-like properties allowing them to subvert innate defenses (Anderson et al., 2003; Justice et al., 2004). The host responds to the pathogenic attack by exfoliation of superficial facet cells, thereby shedding infected cells, and new superficial facet cells begin to form within 72 hr. We characterized the regenerative response of the mouse bladder during acute UPEC infection. Adult (6- to 8-week-old) female C57BL/6 mouse bladders were infected with 10^7 colony forming units (cfu) of the UPEC clinical isolate UTI89 (Mulvey et al., 2001). Mice were sacrificed at 3.5, 6, 12, 24, and 72 hr postinoculation (hpi) for histopathologic analysis.

We found that, in contrast to untreated intact urothelium with large mature superficial cells (Figure 1A), UPEC infection of the bladder led to urothelial hyperplasia within 6 hpi, which was characterized by loss of superficial cells and additional strata of small, immature cells (Figure 1B). At this time point, there was extensive infiltration of acute inflammatory cells, accompanied by hyperemia and edema (normal sequelae of an acute infection) of the lamina propria. The epithelium showed numerous mitotic figures (Figure 1C). All mice were also inoculated intraperitoneally (i.p.) with a nucleotide analog, BrdU, 90 min prior to sacrifice in order to correlate mitotic activity with differentiation state in infected bladders at various time points. The short-pulse BrdU administration labels only cells in the basal layer, and at 6 hpi, a small number of $BrdU^+$ cells can be found (Figure 1D). These $BrdU^+$ cells increased dramatically in number between 12 and 72 hpi (Figures 1E and 1F, $p < 0.05$). $BrdU^+$ cells were also evident in the mesenchymal compartment at 12 hpi (Figure 1G), suggesting activation of this compartment during acute infection. Short-term BrdU labeling of urothelial cells was confined throughout the course of acute infection to the basal layer, where the USCs reside. Together, these results suggest that UPEC infection leads to rapid induction of USC proliferation that then fuels regeneration of sloughed facet cells.

Effect of UPEC Infection on the Bmp4 Signaling Pathway

Our cellular and BrdU labeling studies indicated that the USC niche was activated following bacteria-induced injury to the superficial cells. Previously, we had demonstrated that concerted downregulation of several components of the Bmp4 signaling pathway occurs within 6 hpi in C57BL/6 mice (Mysorekar et al., 2002). Thus, we hypothesized that Bmp4 signaling pathway is a key negative regulator of urothelial turnover. To address the possibility that other Bmp family members may play a role in signaling via *Bmpr1a*, we performed real-time PCR on Bmps 2, 5, and 7 and a key Bmp4 antagonist, Noggin, to determine their baseline bladder expression as well as effects of infection on their expression in wild-type (WT) mice. We found that Bmps 2, 5, and 7 were expressed in the bladder but were not affected by infection at 6 hpi, when Bmp4 shows maximal change. Noggin does not appear to be expressed in the bladder at all (Figure S1). We also examined the expression patterns of additional downstream targets of Bmp4 signaling, namely *TGIF*, $p27^{Kip1}$, and *p63*. *p63*, a p53-related protein, has been demonstrated to be a direct transcriptional target of Bmp signaling (Bakkers et al., 2002), and TGF-β-induced factor (*TGIF*) is a transcriptional corepressor of Smad-mediated transcriptional activation and also a downstream target of Bmp4 (Wotton and Massague, 2001). $p27^{Kip1}$ is a cyclin-dependent

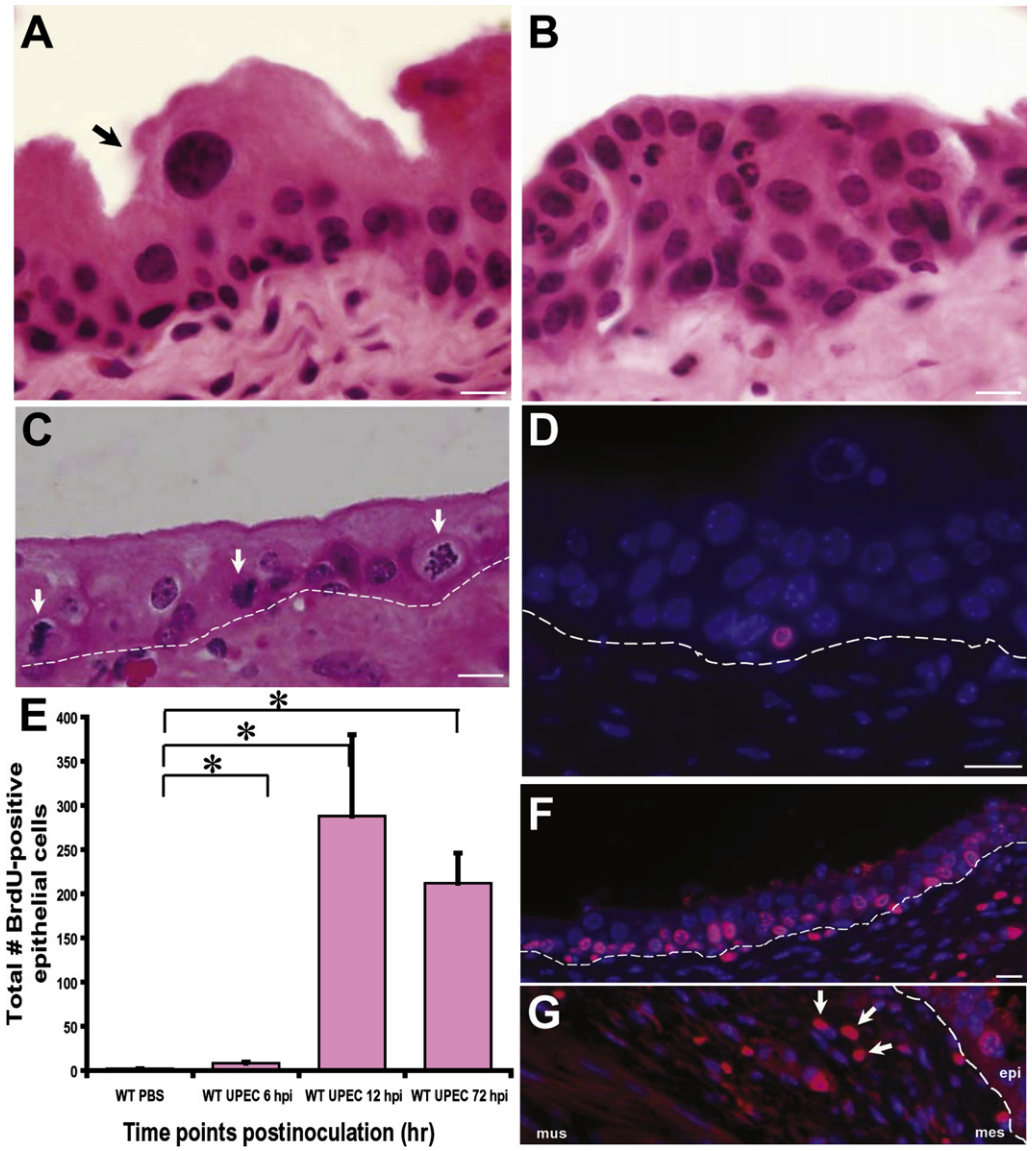


Figure 1. UPEC Infection Accelerates Urothelial Renewal

(A) H&E-stained normal adult urothelium depicting an unperturbed USC niche. Arrows point to a mature superficial cell. Bar = 10 μm.

(B) Twelve hours postinoculation, there is exfoliation of mature superficial cells and hyperproliferation of the underlying immature basal cells concomitant with an inflammatory response.

(C) Numerous mitotic figures (arrows) are evident localized to the basal layer housing presumptive SCs. Dotted lines indicate the epithelial-mesenchymal boundary.

(D) Immunofluorescence (IF) analysis reveals that BrdU (stained red with Alexa Fluor 594-tagged anti-goat secondary antibodies) labels only basal cells at 6 hpi. Nuclei are stained blue with biz-benzimide.

(E) Total number of $BrdU^+$ epithelial cells per tissue section (n = 1 or 2 sections/bladder; 4–7 mouse bladders/time point/condition) were counted. Depicted are data from 6 hr, 12 hr, and 72 hr post UPEC inoculation relative to mock-infected bladders; $p < 0.05$. Bars represent SEM for each group, and p values were computed using a two-tailed Mann-Whitney test.

(F) IF analysis of UPEC-infected bladders at 72 hpi shows numerous $BrdU^+$ cells in the USC niche.

(G) $BrdU^+$ mesenchymal cells are also evident (arrows) in the USC niche at 12 hpi and 72 hpi.

kinase (CDK) inhibitor that suppresses the CDKs active at the G1/S-phase transition in the cell cycle (Glozak and Rogers, 2001; Koff and Polyak, 1995; Polyak et al., 1994a, 1994b). Our results indicate that there is no change in *TGIF* levels upon infection. However, $p27^{Kip1}$ and *p63* were both reduced upon UPEC infection. These effects were specific to activation due to bacterial infection, as they were not induced by PBS mock inoculation. Together, our results suggest that Bmp4 is the key family member playing a role in urothelial homeostasis and renewal.

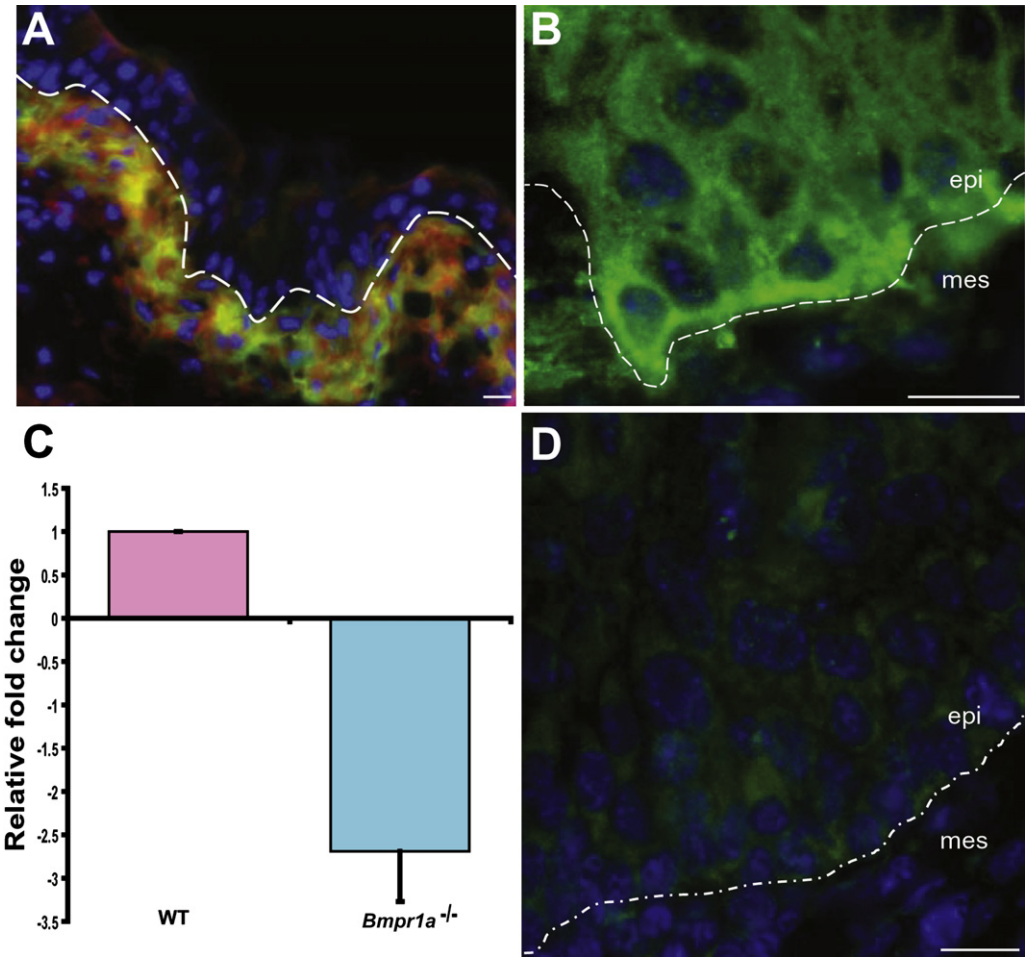


Figure 2. Bmp4 Signaling Pathway Is Active in the Urothelium

(A) IF analysis shows the ligand Bmp4 is localized to the mesenchymal compartment (green with Alexa Fluor 488-tagged secondary antibodies; costained with a mesenchymal marker, Ezh2, red).

(B) Rabbit polyclonal antibodies to *Bmpr1a* (stained green with Alexa Fluor 488-tagged secondary antibodies) reveals that *Bmpr1a* is localized to the basolateral surface of the basal cells in the USC niche.

(C) Quantitative real-time PCR analysis of *Bmpr1a* expression in WT and KO mice, levels decrease upon inducing the KO. Data depicted from 3–4 mouse bladders, analysis was performed in triplicate.

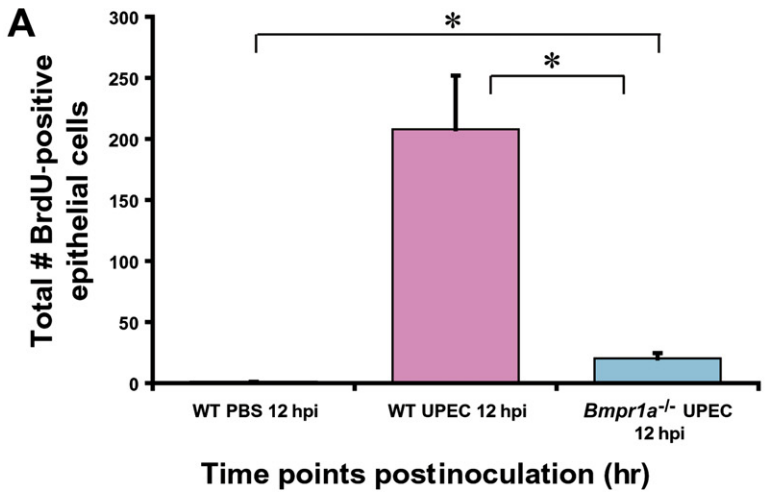
(D) IF analysis indicates Bmpr1a protein is minimally expressed in KO mice. Bar = 10 μm.

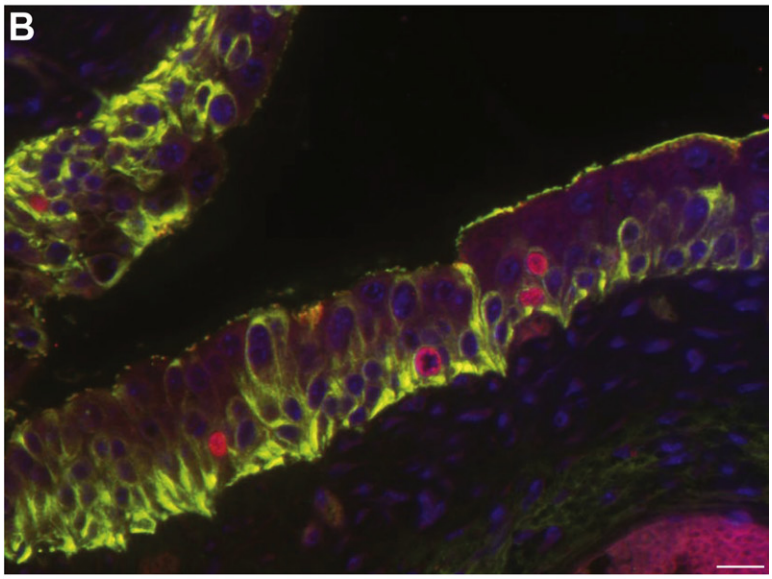
Generation of Mice with Induced Ablation of Urothelial *Bmpr1a* Eliminates Bmp4-Mediated Paracrine Signaling from the Mesenchymal Compartment

To determine the role for Bmp signaling in activation of the USC niche, we first assayed the location of the key signaling pathway components. In untreated bladders, Bmp4 protein itself localized to the mesenchymal compartment (Figure 2A), whereas *Bmpr1a*, the receptor for Bmp4, localized to the epithelium with highest expression, as might be expected, at the basolateral surfaces of the basal progenitor cells (Figure 2B).

Although roles for key signaling pathways are well known in development of tissues, the role of such pathways in adaptation/response to injury is relatively unstudied. To test the hypothesis that Bmp signaling may play a role in the bladder response to infection, we inactivated *Bmpr1a*. Since null *Bmpr1a* mutation leads to embryonic lethality, we used an inducible *Bmpr1a* conditional mutant mouse using a Cre-*loxP* system (Mishina et al., 2002). Mice with the *Bmpr1a* locus flanked by *loxP* sites were mated with inducible β-*Actin-CreER*^{T} mice, which harbor a fusion between Cre recombinase and a mutated hormone-binding domain of the human estrogen receptor (ER^{T}) expressed from the β-actin promoter. Cre-mediated elimination of Bmp4 pathway activity was induced by tamoxifen (TM) injection i.p. into adult *Bmpr1a*$^{fx/fx}$; *CreER*$^{T+}$ mice and control mice at specific times, once a day for a total of three injections. TM treatment activates the modified estrogen receptor, which in turn relocates Cre recombinase into the nucleus to eliminate the floxed *Bmpr1a* alleles.

Mouse bladders were examined histologically prior to TM treatment to verify that there were no effects of unintended ("leaky") Cre expression. We found that untreated *Bmpr1a*$^{fx/fx}$/β-*Actin-Cre*$^{+}$ mice were indistinguishable from β-*Actin-CreER*$^{T+}$; *Bmpr1a*$^{fx/+}$, β-*Actin-Cre*$^{+}$;*Bmpr1a*$^{+/+}$ pups, and their β-*Actin-Cre*$^{-}$ littermates. Upon TM injection, the *Bmpr1a* gene was efficiently





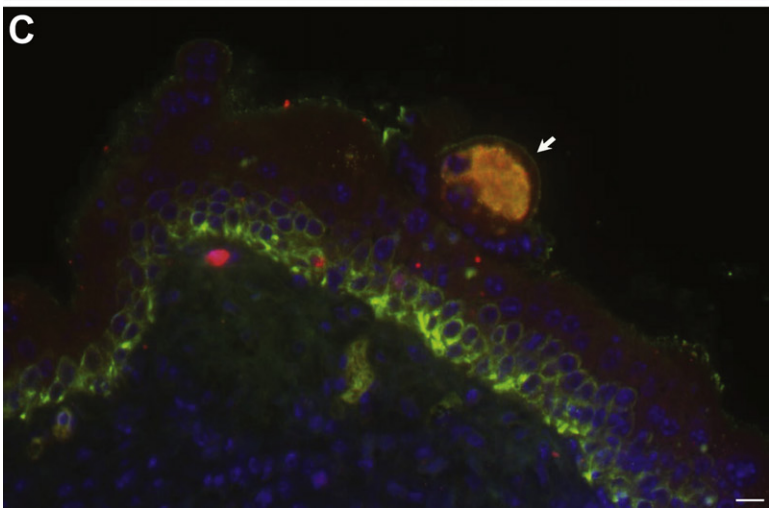


Figure 3. Bmp4 Signaling Regulates Basal Cell Proliferation

(A) Loss of Bmp4 signaling leads to reduced basal cell proliferation. Depicted are counts of $BrdU^+$ cells in 12 hr post UPEC infected WT and KO bladders plus mock-infected bladders. Bars represent SEM for each group. The number of $BrdU^+$ cells in infected *Bmpr1a* KO bladders is markedly reduced relative to infected WT bladders ($p < 0.05$).

(B) IF studies of the KO bladders reveal that $BrdU^+$ (pink) cells are localized to the basal layer ($Cyt5\&6^+$, green staining).

(C) IF studies of *Bmpr1a* KO bladders reveal that IBCs (orange, costained with rabbit antibodies to *E. coli* and BrdU) form normally in superficial cells. $BrdU^+$ epithelial cells (red nuclei) are evident in the basal layer ($Cyt5\&6^+$, green staining). Bar = 10 μm.

targeted as judged by genotyping tail DNA (Figure S2B and Experimental Procedures); however, histological analysis of hematoxylin and eosin (H&E) sections showed, surprisingly, that even 2 weeks after TM induction, the knockout (KO) mice showed no gross defects in urothelial architecture other than slight edema of the lamina propria. These changes were evident in all littermate controls and were likely nonspecific results of TM injection (data not shown).

Genetic ablation of *Bmpr1a* should result in reduction in mRNA and protein expression of the receptor in the KO mouse bladders. Accordingly, we found that *Bmpr1a* expression levels were reduced relative to WT bladders as determined by quantitative real-time PCR (Figure 2C), and the *Bmpr1a* protein was minimally expressed in the KO mice as ascertained by immunostaining (Figure 2D).

Bmpr1a−/− Epithelia Show Markedly Reduced Proliferation in the Basal/SC Compartment at 12 hr Postinfection

We reasoned that since the USC niche is normally quiescent, ablation of Bmp4 signaling may not induce any changes in urothelial architecture in the absence of an activating stimulus. Thus, we induced injury in the bladders of TM-treated $Bmpr1a^{fx/fx}$; $CreER^{T+}$ and heterozygous and WT littermates by inoculation with 10^7 cfu of UTI89 and examined bladders histologically and by immunofluorescence at 6, 12, 24, and 72 hpi. All mice were also inoculated i.p. with BrdU 90 min prior to sacrifice as described before.

We found that complete ablation of Bmp4 signaling led to substantially reduced basal cell proliferation in response to infection at 12 hpi compared to WT bladders (Figure 3A, $p < 0.05$). While the total number of cycling urothelial cells was reduced, they remained localized to the basal layer, as determined by the colocalization of BrdU and Cyt5&6 (Figure 3B). Thus, ablation of Bmp4 signaling leads to an apparent deficiency in basal progenitor cell proliferation immediately following UPEC infection. We also determined that *Bmpr1a* ablation had no detectable effect on the ability of UPEC to undergo IBC formation (Figure 3C). Thus, during the acute stages of infection, UPEC pathogenesis was not dramatically affected in the KO mice.

Inducible Inactivation of *Bmpr1a* Results in a Block in Terminal Differentiation of Superficial Epithelial Cells at 72 hr Postinfection

To further explore the role of Bmp signaling in urothelial renewal, we examined the bladders at 72 hpi, a time of maximal regeneration of superficial facet cells following bacterial-induced damage of the epithelial layers. Overall, the total number of cycling cells at this time point was still reduced relative to infected littermate control bladders (Figure 4A, $p < 0.05$). However, a striking feature emerged in the *Bmpr1a*-deficient bladders at this later time point. There existed an aberrant presence of proliferating cells in the superficial layer: immunofluorescence analysis demonstrated that many superficial cells were still synthesizing DNA (i.e., were $BrdU^+$) (Figure 4B) while concomitantly expressing the terminal differentiation marker uroplakin III (Figure 4C). Basal cells labeled with BrdU were no longer identifiable at this time point, despite their abundant presence in WT controls (Figure 4F). Thus, at 72 hpi in the KO mice, the $BrdU^+$ cells of the bladder (red) were found in the superficial layer of cells, separated from the cytokeratin-positive basal layer (green) (Figure 4D). Normally, in WT mice, these superficial cells are postmitotic, but in the KO mice, they were often in various stages of mitoses (Figure 4E). In WT mice at 72 hpi, only cells in the basal layer are positive for BrdU (Figure 4F), and mitotic activity is evident only in the basal cell layer (Figure 4G, arrow points to a basal layer mitotic figure). Thus, not only was basal cell proliferation reduced overall in the *Bmpr1a*−/− epithelia, but the location of proliferation at 72 hpi was also altered, suggesting that the entire differentiation program was disrupted.

Bmpr1a Ablation Elicits Alterations in Targets of Bmp4 Signaling Pathway

To determine whether targets of Bmp4 signaling were affected by *Bmpr1a* ablation, we examined the expression of *TGIF*, *p63*, and $p27^{Kip1}$. Quantitative real-time PCR detection demonstrated a reduction in these three downstream targets of Bmp signaling in *Bmpr1a*−/− mouse bladders. *TGIF*, *p63*, and $p27^{Kip1}$ were reduced 15-fold, 3-fold, and 7-fold, respectively, in *Bmpr1a*−/− mouse bladders relative to WT mice (Figure 5A). We examined immunolocalization of the $p27^{Kip1}$ protein and found that $p27^{Kip1}$ exhibits high levels of expression in WT bladders, with the highest expression in mature superficial cells (green, Figure 5B), and shows markedly lowered levels in *Bmpr1a* KO bladders (Figure 5C). This reduction in $p27^{Kip1}$ expression may be one underlying mechanism driving the block in terminal differentiation of facet cells. High levels of $p27^{Kip1}$ are known to be critical for a proliferating cell to exit the cell cycle and terminally differentiate. Thus, reduced levels of this protein may contribute to the block in terminal differentiation seen in the *Bmpr1a* KO mice.

Together, our results reveal that ablation of *Bmpr1a* leads to a decrease in Bmp4 pathway activity, indicated by reduced expression of various Bmp4 downstream targets. This altered expression of Bmp4 downstream targets may be responsible, at least in part, for the aberrant renewal in the *Bmpr1a*−/− epithelia.

Requirement for Bmp4 Signaling in Urothelial Renewal Is Specific to Uropathogenic Bacteria and Is Not Induced by an Isogenic Avirulent Mutant

To determine whether USC proliferation and differentiation required bacterial colonization, invasion, and establishment of infection, 21 adult female mice WT, heterozygous, and null for the *Bmpr1a* alleles were infected with an isogenic but avirulent strain of UTI89 (UTI89ΔFimH). FimH is the bacterial adhesin of the type 1 pilus required for bladder colonization, invasion, and IBC formation (Mulvey et al., 1998). UTI89Δ*fimH* does not produce adhesive type 1 pili (Wright et al., 2007). Mice were TM treated as described and infected with UTI89Δ*fimH* for 12, 24, 48, and 72 hr and 7 days. Bladders were isolated from the mice at various time points after infection and analyzed histologically. We found that there was no significant difference in appearance of the UTI89Δ*fimH*-infected bladders relative to the uninfected bladders. In addition, there were no discernible differences between the *Bmpr1a* WT and KO mice. All mice were injected with BrdU prior to sacrifice, and $BrdU^+$ cells were counted at each time point following inoculation. No significant differences above mock infected bladders were seen (data not shown). Thus, the activation of the USC niche was dependent not only on colonization, but upon adherence to or invasion of the urothelium and subsequent activation of the pathogenic cycle described previously (Justice et al., 2004).

Protamine Sulfate-Induced Injury to the Bladder Results in Regeneration that Does Not Activate Stem/Progenitor Cells

Although Δ*fimH* bacteria could not induce USC activation, these mutant bacteria also did not cause exfoliation of superficial facet cells. To determine whether superficial cell exfoliation alone, in the absence of an invasive pathogenic stimulus, was sufficient to induce USC activation, we transurethrally administered protamine sulfate (PS) to adult C57BL/6 mice. We had previously shown that PS induces exfoliation of the superficial facet cells in a dose-dependent manner within the first 12 hr (Mysorekar and Hultgren, 2006). Although an effective superficial cell exfoliant, PS did not induce host inflammation. Superficial cells began regenerating by 48 hr, similar to the time course following infection with UPEC.

To determine the source of the new superficial cells, we performed 90 min BrdU pulse labels, as for UPEC, prior to sacrifice at 1.5, 3.5, 6, 12, 48, and 72 hr post PS treatment. Surprisingly, BrdU was incorporated only by cells above the basal layer at 12 hpi (i.e., suprabasal cells) (Figure 6A). To confirm absence of proliferation in presumptive USCs, we costained cells expressing BrdU with Cyt5&6, which exclusively label basal cells. Our results showed that the $BrdU^+$ cells (red) localized to an intermediate layer and almost never in the basal layer (green) (Figure 6B), suggesting that renewal following PS administration is catalyzed by transient amplifying (TA) cells excluded from the basal layer. In addition, we examined the colocalization between BrdU and uroplakin III (a marker of terminal differentiation). Our results show $BrdU^+$ cells are faintly positive for uroplakin expression (Figure 6C). These levels are consistent with the cells being of intermediate character (i.e., neither basal nor superficial cells). We also observed $BrdU^-$, mature superficial facet cells (arrow, Figure 6D) with high and dense expression of uroplakin III, which were nearby $BrdU^+$ intermediate cells. Quantification of the $BrdU^+$ cells revealed that at 12 hpi, there were numerous dividing cells, and these numbers decrease dramatically by 72 hr (Figure 6E, $p < 0.05$). Interestingly, despite the frequent mitotic activity of the suprabasal cells, the epithelium is not hyperplastic

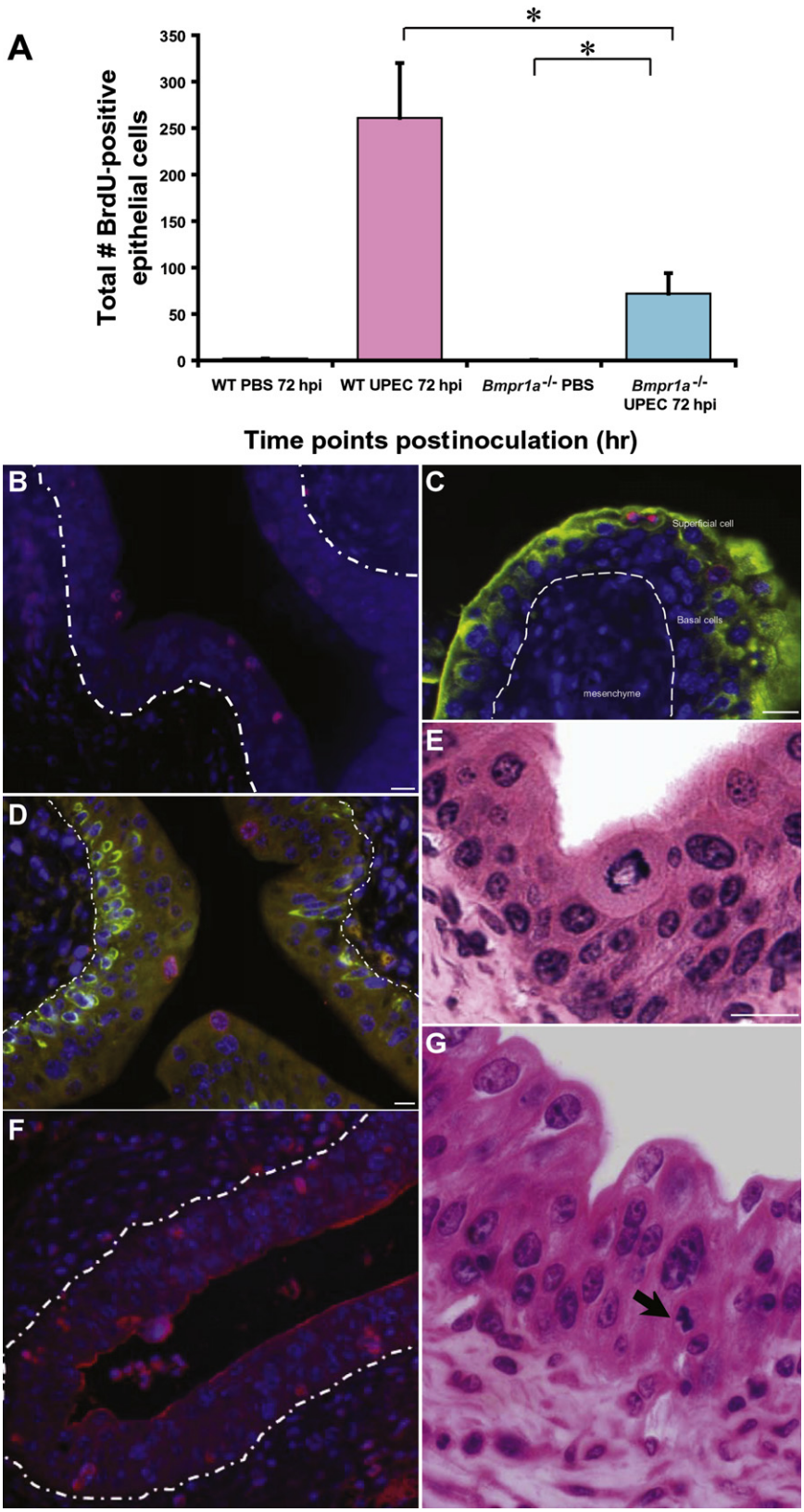


Figure 4. Bmp4 Signaling Is Necessary for Terminal Differentiation of Superficial Facet Cells

(A) *Bmpr1a*−/− bladders 72 hpi show reduced proliferation relative to infected WT control bladders ($p < 0.05$). Depicted are total numbers of $BrdU^+$ epithelial cells/section (n = two sections/bladder, 4–7 mouse bladders/time point/condition) from 72 hr post UPEC infection of WT and KO bladders and from mice mock inoculated with PBS. Bars represent SEM for each group.

(B) All $BrdU^+$ (pink) nuclei are in the luminal/superficial layer at 72 hpi. Bar = 10 μm.

(C) IF studies of the KO bladders at 72 hpi reveal that the $BrdU^+$ (pink) cell is also uroplakin$^+$ (green).

(D) IF of 72 hr UPEC infected *Bmpr1a*−/− bladders with cells stained with BrdU (pink) and Cyt5&6 and 14 (basal cell markers, stained green). No colocalization is evident.

(E) H&E-stained section of a bladder from 72 hr UPEC infected *Bmpr1a* KO mouse shows a cell undergoing mitosis in the superficial layer while exhibiting differentiated cell characteristics.

(F) BrdU staining of 72 hr UPEC-infected WT bladders reveals $BrdU^+$ cells localizing to the basal cell layer only.

(G) H&E-stained 72 hr UPEC-infected WT bladder shows mitotic cell localized to basal layer.

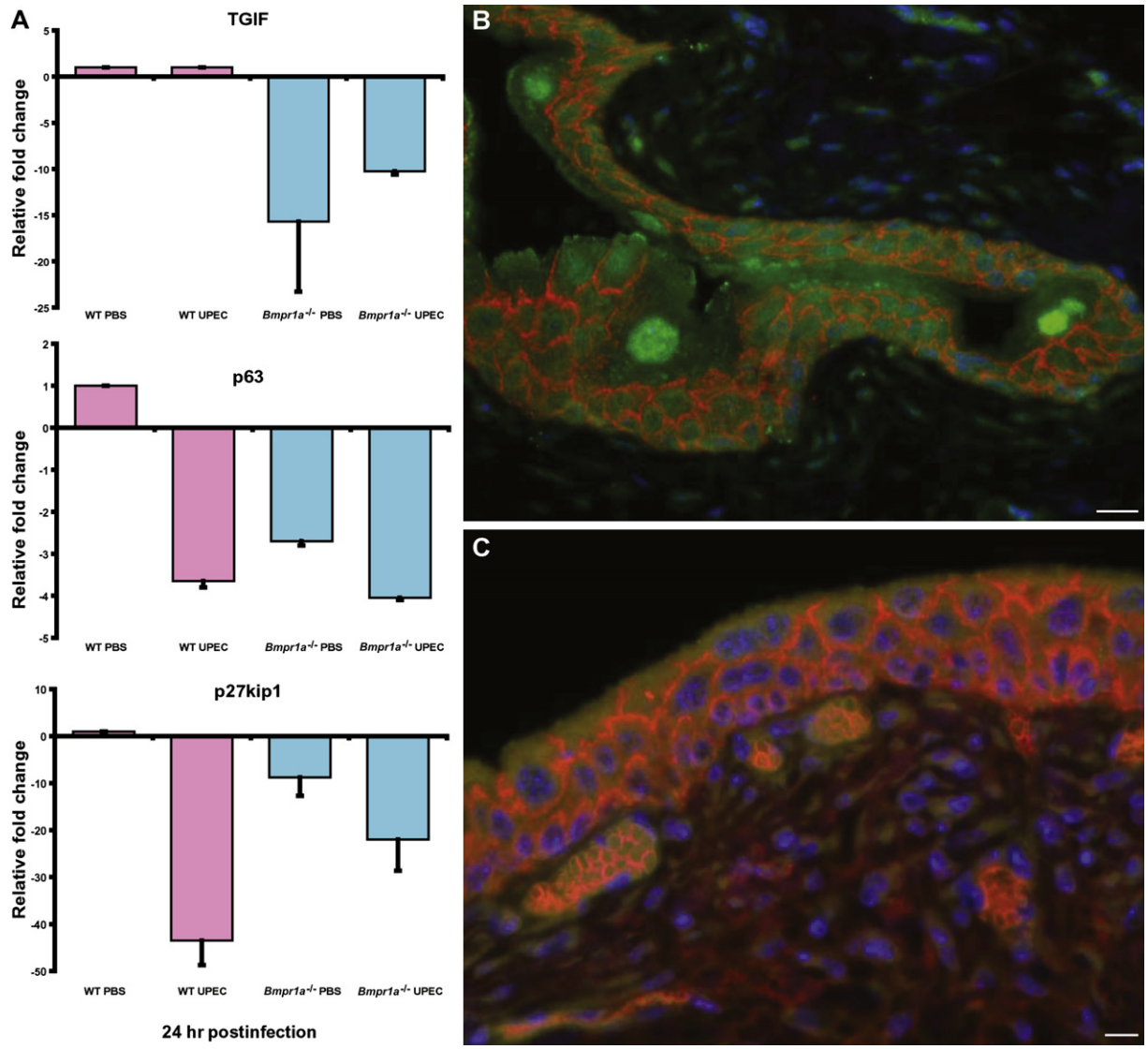


Figure 5. Bmp4 Pathway Activity Is Altered in *Bmpr1a* KO Bladders

(A) Quantitative real-time PCR analysis of fold changes in bladder expression of Bmp4 targets, *p63*, *TGIF*, and $p27^{Kip1}$ at 24 hr following inoculation of UPEC. Mean values ± SEM are plotted for three independent assays of RNAs, each pooled from three or four animals. mRNA levels were first normalized to 18S rRNA, which served as an internal reference control. The normalized values were then referenced to levels of the transcript in bladder RNA prepared from mock-inoculated mice. Levels in control bladder RNA are arbitrarily set at 1.

(B) IF analysis shows high levels of $p27^{Kip1}$ expression (stained green with Alexa Fluor 488-tagged secondary antibodies) in WT bladders, with highest expression in mature superficial facet cells and minimally present in the basal and intermediate cells (E-cadherin-stained red/orange with Alexa Fluor 594-tagged secondary antibodies).

(C) *Bmpr1a*−/− bladders show markedly reduced levels of $p27^{Kip1}$ expression. Bar = 10 μm.

(Figure 6F), and as expected, there was no evidence of accumulation or infiltration by inflammatory cells (Figure 6G). Even at 72 hpi, a few presumptive TA cells remain positive for BrdU, although they continue to be localized to the suprabasal layer adjacent to newly regenerated mature superficial cells (arrows, Figure 6H).

The above results argue that renewal following PS administration is mediated by cells of intermediate differentiation or TA cells and not the basal USCs. To further illustrate the marked difference in the bladder response to inflammation-inducing injury (e.g., infection with UPEC) compared to chemical, noninflammatory injury, we used Affymetrix GeneChips to assay global expression profiles (Doherty et al., 2008; Lugus et al., 2007). We compared global gene expression profiles in the bladders of PS-treated versus UPEC-infected C57BL/6 mice. We found that the gene-expression changes induced by PS at time points <6 hr were dramatically different from those induced by UPEC infection (Figure S3). Thus, at time points in which infection is known to induce marked changes in the expression of cell cycle and inflammation-related genes, a completely different pattern

of gene expression was induced by PS treatment. Thus, the molecular regulation of urothelial response to chemical injury is distinct from the response to infectious injury.

Chemical Injury to the *Bmpr1a*−/− Bladders Did Not Induce Aberrant Renewal

We investigated whether inactivation of Bmp4 signaling affected response to chemical injury, since UPEC infection seemingly led to Bmp4-dependent activation of the USC niche. If the PS-induced bladder response is independent of Bmp4, then we hypothesized that inoculation of *Bmpr1a*−/− mouse bladders with PS should not have a response different from WT or heterozygous littermates. Mice were treated with TM once daily for 3 days and inoculated with 10 mg/ml PS for 12 hr, 48 hr, 72 hr, and 2 weeks. Bladders were isolated from the mice after inoculation and analyzed histopathologically by H&E staining of bladder sections, presence or absence of epithelial hyperplasia, mitotic activity, and quantitation of $BrdU^+$ cells at each time point. We did not detect any discernible differences between the KO and heterozygous mice at 12 or 72 hr (data not shown). Our results further provide evidence that PS treatment does not activate the USC niche and that urothelial regeneration from PS-mediated injury does not require the Bmp4 pathway.

DISCUSSION

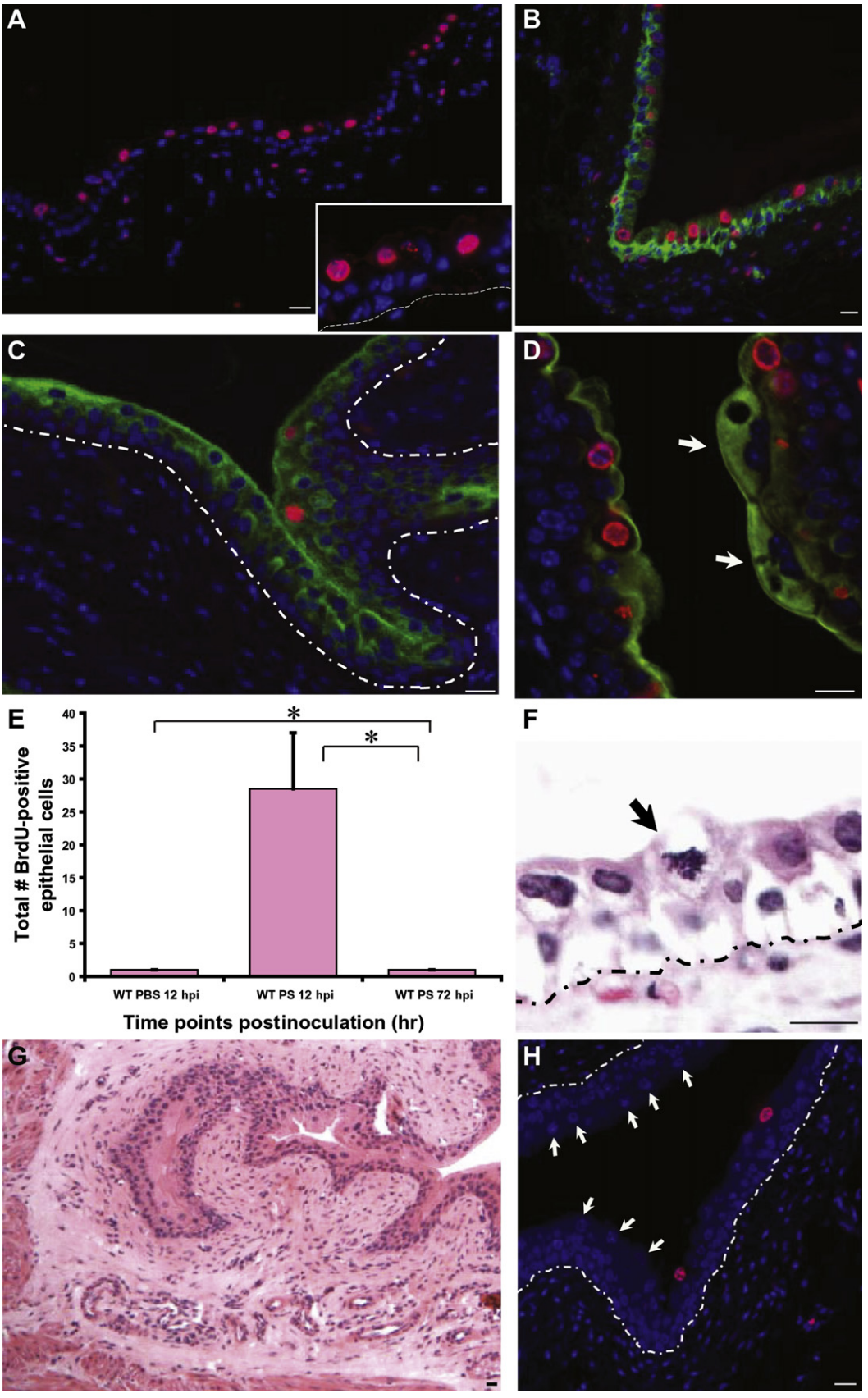
Modulating Bmp Signaling to Balance Quiescence and Activation of USC Niche in Response to UPEC Infection

Our previous data (Mysorekar et al., 2002) showed that urothelial basal cells normally display signs of activated Bmp signaling in vivo; however, unlike the hair follicle, where ablation of Bmp4 pathway activity induced the follicular SCs to precociously enter the proliferative phases associated with the new hair cycle (Kobielak et al., 2007), our current results show that ablation of Bmp signaling alone was not sufficient to disrupt the quiescent state of the USC niche in the bladder. Instead, our loss-of-function studies highlighted an essential role for Bmp signaling in maintaining the responsiveness of the USC niche, because in its absence, USC proliferation was reduced 100-fold in response to infection. Further, our studies demonstrate that Bmp signaling is required for proper terminal differentiation following UPEC infection. The UPEC pathogenic cycle includes exfoliation of the superficial facet cells and subsequent basal cell proliferation, and upon resolution of infection, the terminally differentiated facet cell layer is replaced, thus restoring the impermeable uroplakin barrier that protects the bladder epithelium from the contents of the bladder lumen. However, in the absence of *Bmpr1a*, induction of basal cell proliferation by infection and the regenerative response was severely hampered. Intriguingly, superficial cells continued to divide and failed to undergo terminal differentiation in the KO mice. Thus, we propose a model whereby the USC niche may be defined as comprising the proliferative basal epithelial cell compartment and the underlying mesenchyme. In normal unperturbed bladders, Bmp signaling from the mesenchyme may be required to maintain both USC quiescence and terminal differentiation of superficial cells. UPEC infection triggers epithelial cell damage and inflammation, resulting in the modulation of this key pathway in order to trigger exit from quiescence (i.e., to fuel basal cell proliferation), to induce their terminal differentiation into superficial cells, and to restore normal physiological barriers. Ongoing studies in the laboratory suggest that the absence of normal patterns of renewal due to ablation of Bmp signaling predisposes to a chronic inflammatory condition characterized by lack of superficial cells, changes in tissue architecture, and luminal bacterial colonization (data not shown). Investigations aimed at defining the molecular and cellular nature of the observed abnormalities in long-term pathogenesis of UPEC-infected *Bmpr1a*−/− mice are in progress.

Bmp4 Signaling Is Essential for Urothelial Regeneration and Repair in Response to Infectious, but Not Chemical, Injury

In the current study, we have elucidated the urothelial regenerative response to infection and use the currently available USC markers (e.g., BrdU labeling, position within the epithelium, Cyt5&6, and BrdU colocalization) to characterize the cellular bases of the regenerative response. We have shown for the first time that the pattern of cellular and molecular response of the urothelium to chemical injury differs markedly from its response to infectious injury. These differences are seen despite similarities in the kinetics and magnitude of facet cell exfoliation and urothelial regeneration in both types of injury. Injury induced by the basic cationic protein PS did not induce basal cell proliferation or change molecular aspects of the underlying USC niche, despite facet cell exfoliation and epithelial regeneration of similar time course and magnitude as followed UPEC inoculation. Rather, PS treatment led to activation and proliferation of cells of intermediate differentiation residing above the basal level and led to no change in Bmp4 pathway activation. Thus, chemical agents may cause exfoliation, but not inflammation, and may induce TA cells, rather than USCs, to mediate repair of superficial cell damage. These studies have the caveat that SCs likely constitute a subset of these basally located $Cyt5\&6^+BrdU^+$ cells; molecular markers entirely specific for the SC remain to be defined, and as yet there are no clonogenic assays that functionally prove stemness in the urothelium.

Ablation of Bmp signaling did not alter the regenerative response to PS-mediated injury. Bmp4 signaling is apparently dispensable for urothelial renewal that does not involve proliferation of basally located, presumptive urothelial SCs, because exfoliation/regeneration-inducing injury by chemical treatment and by nonadherent or nonpathogenic bacteria does not require Bmp4. The different requirements for Bmp4 may reflect that mesenchymal Bmp4 elaboration may depend on inflammation-mediated signals. GeneChip and histological studies demonstrated that PS-treated bladders have minimal inflammation compared to UPEC-infected bladders. Thus, chemical agents that cause exfoliation but not inflammation may induce TA cells rather than USCs to mediate repair of superficial cell damage. Our results suggest that exfoliation inducing injury in the absence of inflammation leads to bladder regeneration based almost exclusively on TA cell amplification. Recent work has stressed the difference between lineage-specific SCs and TA cells, which carry the major proliferative duties of epidermis (Kobielak et al., 2003, 2007). Further studies aimed at dissecting specific roles for various immune-cell populations in modulating the USC niche will shed light on the cells that might mediate the changes in Bmp signaling required for SC activation.


A
B
C
D
E
F
G
H
Total # BrdU-positive epithelial cells
40
35
30
25
20
15
10
5
0
*
*
WT PBS 12 hpi
WT PS 12 hpi
WT PS 72 hpi
Time points postinoculation (hr)

Significance of Bmp4 Signaling in Human Disease

Bmp signaling has emerged as a common pathway for controlling SC self-renewal and lineage fate from *Drosophila* to mammals. However, this is the first time that Bmp signaling has been implicated in progression of an acute infection process in any tissue. Differences in regulation of urothelial renewal in humans may influence progression of UTIs (whether they are acute and self-limited or can become chronically recurring). Previously, we showed that UPEC can persist indefinitely within the immature basal cells enclosed within lysosomal compartments and can re-emerge from these quiescent reservoirs to seed recurrent UTIs (Mysorekar and Hultgren, 2006). Our study had suggested that UTI recurrence may depend on UPEC's ability to manipulate urothelial differentiation/proliferation in the bladder, and that stem and early progenitor cells serve as a protective niche for UPEC to escape immune detection and evade exfoliation. Dysregulation of urothelial renewal by inactivation of Bmp signaling could affect both formation of UPEC intracellular reservoirs and their re-emergence from this state concomitant with proliferation/differentiation cascades. Our findings may also have clinical significance for another major chronic disorder that is of low incidence and unknown etiology affecting bladders of women: interstitial cystitis (IC), characterized by damaged urothelial barriers, including loss of a superficial facet cell layer and abnormally permeable membranes (Parsons, 2007). Indeed, aberrations in Bmp4 pathway activity could be one genetic etiological factor leading to certain women being more susceptible to IC. Thus, recombinant activators of Bmp4 signaling could be used to modulate activation of the USC niche and to induce terminal differentiation and restoration of normal physiologic barriers. Understanding how the urothelium renews itself following infection may be critical not only for understanding of acute and recurrent UTIs, but also for understanding another major malignancy: bladder cancer, which is characterized by aberrant urothelial turnover and chronic inflammation. Future studies may demonstrate how aberrations in important bladder signaling pathways regulating urothelial proliferation, like the Bmp4 pathway, may predispose patients to recurrent UTIs, IC, or the aberrant proliferative and differentiation patterns that result in bladder cancers.

Prospectus

Together, our study distinguishes two pathways of epithelial injury response in the same tissue. One, the response to infection, involves activation of basal SCs and is regulated at the molecular level by Bmp4 signaling. The other, response to non-inflammatory, exfoliative chemical injury, appears to involve increased proliferation of TA cells. The molecular regulation of this latter pathway remains to be elucidated but clearly does not seem to depend on Bmp4. Future studies will elucidate the potential role of inflammatory mediators in regulating Bmp4 signaling, as well as the principal mediators of noninflammatory injury response, and might eventually shed light on how the bladder responds to chronic inflammation (e.g., IC) and how it might progress to development of bladder cancer.

EXPERIMENTAL PROCEDURES

Mice

Experiments were performed using protocols approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381-01). Mice were maintained under specified pathogen-free conditions in a barrier facility and under a strict 12 hr light cycle.

Bacterial Strains

UTI89 (Mulvey et al., 2001), a pathogenic UPEC strain recovered from a patient with UTI (Langermann et al., 2000), was transformed with a pCOM-GFP plasmid (Valdivia et al., 1996) and grown for 16 hr in Luria Broth as a static culture at 37°C. Bacterial strains were grown using standard techniques. UTI89Δ*fimH* (Wright et al., 2007) contains a deletion of the *fimH* adhesin (type1$^+$/FimH$^-$).

Inoculations of Mice

Mice were anesthetized and inoculated via transurethral catheterization with 50 μl of bacterial suspension (10^7 cfu/ml) in phosphate-buffered saline (PBS) as previously described (Hannan et al., 2008; Mysorekar and Hultgren, 2006). At the indicated times, mice were sacrificed, and their bladders were aseptically removed and processed for microscopy, histology, and cfu titration. All analyses were performed in the urothelia of adult female mice (n = 4–7 mice per experimental group for all experiments, n = 1–2 experiments).

Protamine Sulfate Treatment

PS (Sigma) was delivered transurethrally at a concentration of 10 mg/ml in water (Mysorekar and Hultgren, 2006). All analysis was performed in the urothelia of adult female C57BL/6 mice (n = 4–7 mice per experimental group for all experiments, n = 1–2 experiments).

Histochemical and Immunofluorescence Analysis

For histological and immunofluorescence studies, bladders were dissected into two halves and incubated in 10% formalin overnight at 4°C. The bladder tissues were embedded in 2% agar for paraffin processing. For immunohistochemical analysis, 4–5 μm serial sections were cut longitudinally, deparaffinized in fresh xylene (2 × 10 min, RT), rehydrated in isopropanol (3 × 5 min, RT), antigen retrieved by boiling for 30 min in 10 mM NaCitrate buffer, blocked in 1% BSA/0.3% Triton X-100 for 1 hr at RT, and subsequently incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: (1) rabbit polyclonal antibodies to *E. coli* (1:500) (United States Biological; Swampscott, MA), (2) rat anti-mouse E-cadherin (1:500) (Zymed; San Francisco),

Figure 6. PS Treatment Activates Transient Amplifying Cell Populations

(A) IF analysis reveals that BrdU (stained red with Alexa Fluor 594-tagged anti-goat secondary antibodies) labels nonbasally located (intermediate) cells (shown in higher magnification in [A] inset). Nuclei are stained blue with biz-benzimide. Dotted lines indicate the epithelial-mesenchymal boundary.

(B) BrdU$^+$ cells (red) do not colabel with the Cyt5&6$^+$ basal urothelial cells (stained green).

(C) IF analysis of a 12 hr PS-treated bladder, showing limited colocalization between BrdU (red) and uroplakin III, a terminal differentiation marker (stained green with Alexa Fluor 488-tagged anti-mouse secondary antibodies).

(D) Higher magnification with a mature, multinucleated superficial facet cell densely expressing uroplakin III (arrow, green staining) opposite several BrdU$^+$ intermediate cells.

(E) Total number of BrdU$^+$ epithelial cells was counted in 12 hr and 72 hr post PS-treated bladders (n = 1–2 sections/bladder; 4–7 mouse bladders/time point/condition). Bars represent SEM for each group.

(F) H&E-stained PS-treated bladders showing a mitotic figure in the suprabasal layer of the urothelium (arrow).

(G) H&E-stained 12 hr PS-treated WT bladder, showing no evidence of an inflammatory infiltrate.

(H) IF analysis of BrdU$^+$ cells in PS-treated WT bladders, PS treated for 72 hr, with few proliferating cells in suprabasal layer with presence of numerous regenerated mature facet cells present (arrows). Bar = 10 μm.

(3) mouse monoclonal antibody (mAb) to uroplakin III (Research Diagnostics; Concord, MA), (4) mouse mAb to Cyt5&6 (clone D5/16B4, Chemicon; Billerica, MA) (1:500), (5) goat polyclonal antibody to *Bmpr1a* (R&D Systems; Minneapolis), and (6) mouse monoclonal antibody to Bmp4 (Novocastra; Newcastle, UK and R&D Systems). After three PBS washes (3 × 5 min at RT), antigen-antibody complexes were detected with Alexa Fluor 488, 594, and 647-conjugated secondary antibodies (1:500) (Invitrogen; Carlsbad, CA).

SYBR Green-Based Quantitative Real-Time PCR

Age-matched female WT C57BL/6 mice or *Bmpr1a*−/− mice were infected with UTI89 strain for multiple time points (n = 4 animals/time point/strain). Bladder RNAs were pooled as above and used for quantitative real-time PCR studies. cDNAs were added to 25 μl quantitative real-time PCRs containing 12.5 μl of 2× SYBR Green Master Mix (Applied Biosystems; Foster City, CA) and 900 nM gene-specific primers. A melting curve was used to identify a temperature where only the amplicon and not primer dimers accounted for the SYBR Green-bound fluorescence. Assays were performed in triplicate with a Bio-Rad MyiQ instrument. All data were normalized to an internal standard (18S rRNA; ΔΔCT method).

Generation of *Bmpr1a* KO Mice

In the *Bmpr1a*$^{fx/fx}$ mouse line, the second exon of the *Bmpr1a* gene is flanked by two *loxP* sites (Mishina et al., 2002). Using the *Bmpr1a*$^{fx/fx}$ line and the TM-inducible Cre line β-*Actin-CreER*^{T} (Jackson Laboratory), β-*Actin-Cre* + *Bmpr1a*$^{fx/fx}$ homozygous, β-*Actin-Cre* + *Bmpr1a*$^{fx/+}$ heterozygous, and β-*Actin-CreER*$^{T-}$ *Bmpr1a*$^{fx/fx}$, β-*Actin-CreER*$^{T-}$ *Bmpr1a*$^{fx/+}$, or WT control mice were generated. Offspring yielded litters of the expected numbers, genotype, and Mendelian ratios (Figures S2A and S2B). To introduce *Bmpr1a* inactivation, adult female mice were i.p. injected with 25 mg/ml TM (Sigma) at specific time points, once every day for a total of three injections. TM induces Cre expression. Cre recombinase in turn mediates *loxP*-dependent DNA recombination.

BrdU Labeling

To determine mitotic activity, all mice were injected i.p. with an aqueous solution of 5-bromo-2′-deoxyuridine (120 mg/kg) and 5′-fluoro-2′-deoxyuridine (12 mg/kg) (Sigma) 90 min prior to sacrifice. All BrdU count analyses were performed on two separate sections, each containing bisected bladders from 4–7 adult female mouse bladders/time point/condition.

Statistical Analysis

Statistical analysis was performed using Mann-Whitney U test (two-tailed). A value of $p < 0.05$ was considered to be significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and three figures and can be found online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00106-1.

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